Survivin selective inhibitor YM155 promotes cisplatin-induced apoptosis in embryonal rhabdomyosarcoma

TAKEHISA UENO, SHUICHIRO UEHARA, KENGO NAKAHATA and HIROOMI OKUYAMA

Pediatric Surgery, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan

Abstract. Survivin, a member of the inhibitor of apoptosis protein family, functions as a key regulator of programmed cell death. YM155 is a small molecule that selectively inhibits survivin. We investigated the effect of YM155 on survivin suppression in the human rhabdomyosarcoma (RMS) cell line RD. The efficacy of YM155 in combination with cisplatin was also determined in a xenograft model. The effect of YM155 on survivin expression in the RD cell line was examined at both mRNA and protein levels using real-time PCR and western blot analysis. RD cells were cultured with various concentrations of YM155, then cisplatin was added to the medium and the anti-proliferation response was determined. Cell growth was evaluated by WST-8 assay. Finally, the efficacy of YM155 combined with cisplatin was examined in an established xenograft model. Survivin mRNA levels in the RD cell line were decreased to 72 and 24% at 24 and 48 h, respectively, after 10 nM of YM155 was added. YM155 also decreased the levels of survivin protein. YM155 treatment (10 nM) inhibited cell proliferation of RD in a dose-dependent manner *in vitro*, with 58% of cells viable at 48 h. When cultured with 10 nM of YM155 and 10 μM cisplatin, RD cells demonstrated only 25% of the growth observed when cultured with cisplatin alone. YM155 in combination with cisplatin significantly inhibited tumor growth by 13% compared with control (P<0.0001) in RD xenograft tumors. YM155 increased the sensitivity of cisplatin by suppressing survivin in the embryonal RMS cell line RD. Further studies should investigate the use of YM155 as an apoptosis inducer, either alone or in combination with cisplatin, for the treatment of malignant RMS.

Correspondence to: Dr Takehisa Ueno, Department of Pediatric Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
E-mail: ueno@pedsurg.med.osaka-u.ac.jp

Abbreviations: RMS, rhabdomyosarcoma; PCR, polymerase chain reaction; FACS, fluorescence activated cell sorting; TUNEL, terminal deoxynucleotidyl transferase dUTP-biotin nick end-labeling

Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in children, the incidence of RMS in children <20 years old is 4.3 per million per year (1). Current treatment for RMS relies on chemotherapy, along with surgery and radiotherapy. The prognosis for non-metastatic RMS has improved considerably over the last 30-40 years due to use of combination chemotherapy (2-4). Despite significant improvements in survival over the past few decades, more than one-third of RMS patients continue to die of the disease (5). In particular, the outcomes of patients with metastatic disease remain very poor, despite intensification of treatment with agents known to be active and attempts at introducing novel therapies (6-8). The mainstay of chemotherapy for RMS continues to be vincristine and actinomycin D in combination with cyclophosphamide, so-called VAC treatment. The most recent Children's Oncology Group (COG) trial for low-risk RMS showed that patients have an excellent outcome [2-year event-free survival (EFS), 88%; overall survival (OS), 98%] with short therapy duration (9). Whereas the most recent COG high-risk RMS study showed poor outcome in 3-year EFS 38% (10).

Other drugs such as doxorubicin, etoposide, and cisplatin have been added to this combination in higher risk patients, but to date there is no definitive proof of improved outcomes with the addition of these drugs, despite evidence of response in the relapse setting (6).

Survivin is a 16.5-kDa member of the inhibitor of apoptosis protein family, and blocks the mitochondrial pathway of apoptosis by inhibiting caspases (11). Survivin is not expressed in differentiated normal tissue (12,13). Higher survivin expression has been correlated with unfavorable survival or disease recurrence in various adult malignancies (12,14-22). In the pediatric setting, most primary hepatoblastoma tissue specimens express survivin, and its expression was shown to increase after chemotherapy, suggesting that survivin may play a role in the survival of tumor cells (23). Survivin has therefore become a therapeutic target of pediatric tumors.

YM155 is a novel, small, imidazolium-based compound that specifically inhibits survivin, as determined using a survivin promoter activity assay (24). In pre-clinical experiments, YM155 as a single agent downregulated the transcription of survivin in a dose/time-dependent manner, triggering p53-independent apoptosis in a wide range of

human tumor cells. YM155 also induced tumor regression in established cancer xenografts (24,25), and the combination of YM155 with various chemotherapeutic agents potentiated apoptosis induction in several human cancers (26-28). Despite its demonstrated efficacy in targeting tumor cells, the effects of YM155 in combination with DNA-damaging drugs have remained largely unknown.

Once primary chemotherapy fails, cancer cells became resistance to the drug. We used a cell line that was treated by cyclophosphamide, therefore other DNA-damaging drugs should be explored (29).

The platinum compound cisplatin, DNA damaging drug, showed efficacy combined with YM155 in previous reports (28,30,31). In this study, we selected cisplatin combined with YM155 to search for a new regimen. We examined the effects of YM155 in combination with the platinum compound cisplatin on a human RMS cell line both *in vitro* and *in vivo*. Given the importance of survivin in cisplatin apoptosis resistance, and with the aim of finding new strategies to reduce cisplatin doses in patients with RMS, we sought to determine whether YM155 synergized with cisplatin to affect the RMS cell line *in vitro,* and as a tumor xenograft.

Materials and methods

Cell culture. Cells from the human rhabdomyosarcoma cell line RD were obtained from Japanese Collection of Research Bioresources Cell Bank. Cells were maintained in Gibco RPMI-1640 (Thermo Fisher Scienrific, Waltham, MA, USA), supplemented with 10% heat-inactivated fetal bovine serum in a humidified incubator with 5% CO_2 at 37°C.

Reagents. YM155 was purchased from Selleck Chemicals (Houston, TX, USA) and dissolved with DMSA to a final stock solution concentration of 1 mM cisplatin (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in sterile water to a final stock solution concentration of 1 mM.

Real-time PCR. RD cells were cultured in medium with or without various concentrations of YM155 and/or cisplatin. Control cultures received dimethyl sulfoxide 0.1%. For RNA extraction, NucleoSpin RNA (Takara, Osaka, Japan) was used according to the manufacturer's protocol. Primix Taq (Takara) was used for the reaction. The primers to detect human Survivin (Invitrogen, Carlsbad, CA, USA) used for PCR were as follows: 5'-GGCTCTTTCTCTGTCCAGTT-3' (forward), 5'-ACCACCGCATCTCTACATTC-3' (reverse). Equal volumes of PCR products were electrophoresed through agarose gel in TBE buffer.

Quantitative PCR. Custom primers to detect human survivin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were created by Invitrogen. Sybergreen analysis was carried out with SYBR Premix Ex Taq II (Takara). Quantitative PCR was performed using an Applied Biosystems ViiA 7 System (Thermo Fisher). Results from each sample were compared using the comparative CT method (Applied Biosystems). Genomic levels and cDNA expression levels were measured relative to GAPDH. Experiments were performed in triplicate.

Western blot analysis. Protein was extracted by western blot assays. Extracted protein samples were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking at room temperature with TBS-T buffer, each membrane was incubated for 1 h at 4°C with the primary antibodies anti-survivin (Cell Signaling, Beverly, MA, USA) and mouse anti-β-actin (Cell Signaling). The membranes were then washed with TBS-T and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunostar Zeta (Wako Pure Chemical Industries) was used as luminescence solution. Proteins of interest were visualized using the ImageQuant LAS 4000 Mini (General Electric Co., Fairfield, CT, USA).

RNA interference. Validated human anti-survivin siRNA was purchased from Life Technologies Corp. (Carlsbad, CA, USA). A control scramble (nonsilencing) siRNA was purchased from Cell Signaling Technology. Cells were seeded at 24 h before transfection with either anti-survivin siRNA or control scramble siRNA using the Lipofectamine RNAiMAX Transfection reagent (Life Technologies Corp.). The final concentration of siRNA duplex in the culture medium was 50 nmol/l.

Cell growth inhibition assay. The RD cell lines were used to determine the inhibitory effect of YM155 on cell growth by the WST assay, which uses the highly water-soluble tetrazolium salt WST-8. Cells ($6x10^4$/well) were plated in 96-well plates and then cultured in medium with or without various concentrations of YM155 and/or cisplatin. Control cultures received 0.1% DMSO. At 24 and 48 h, after the treatment, the percentage of viable cells in each well was examined with a Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) using a Multiscan Go microplate multi-spectrophotometer (Thermo Fisher Scientific).

Annexin V assays. Annexin V/propidium iodide staining was carried out using a BD Annexin-V kit (BD Biosciences, Franklin Lakes, NJ, USA), and analyzed by FACS in a BD FACS Canto II flow cytometer (BD Biosciences).

Assay of caspase-3 activity. Sixty thousand cells from each experimental condition were assayed for caspase-3 activity using the Apo-ONE Homogeneous Caspase-3/7 assay (Promega, Madison, WI, USA). This system uses the profluorescent substrate rhodamine 110 bis-(N-CBZL-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110), which allows highly sensitive, quantitative measurement of caspase-3 protease activity. Caspase activity, measured with the SH-9000 microplate reader (Corona Electronic, Hitachinaka, Japan), is indicated by intense rhodamine 110 fluorescence at 499 nm. The emission maximum is 521 nm. Experiments were performed in triplicate.

In vivo antitumor activities in an RD xenograft model. Four-week-old male mice (NOD SCID) were purchased from Oriental Yeast (Tokyo, Japan). RD cells ($2x10^6$) were injected into the backs of the mice. After tumors grew to >20 mm in diameter, 5-mm cubic sections of tumor were transplanted into the flank of 4-week-old male nude mice (BALB/c nu/nu),
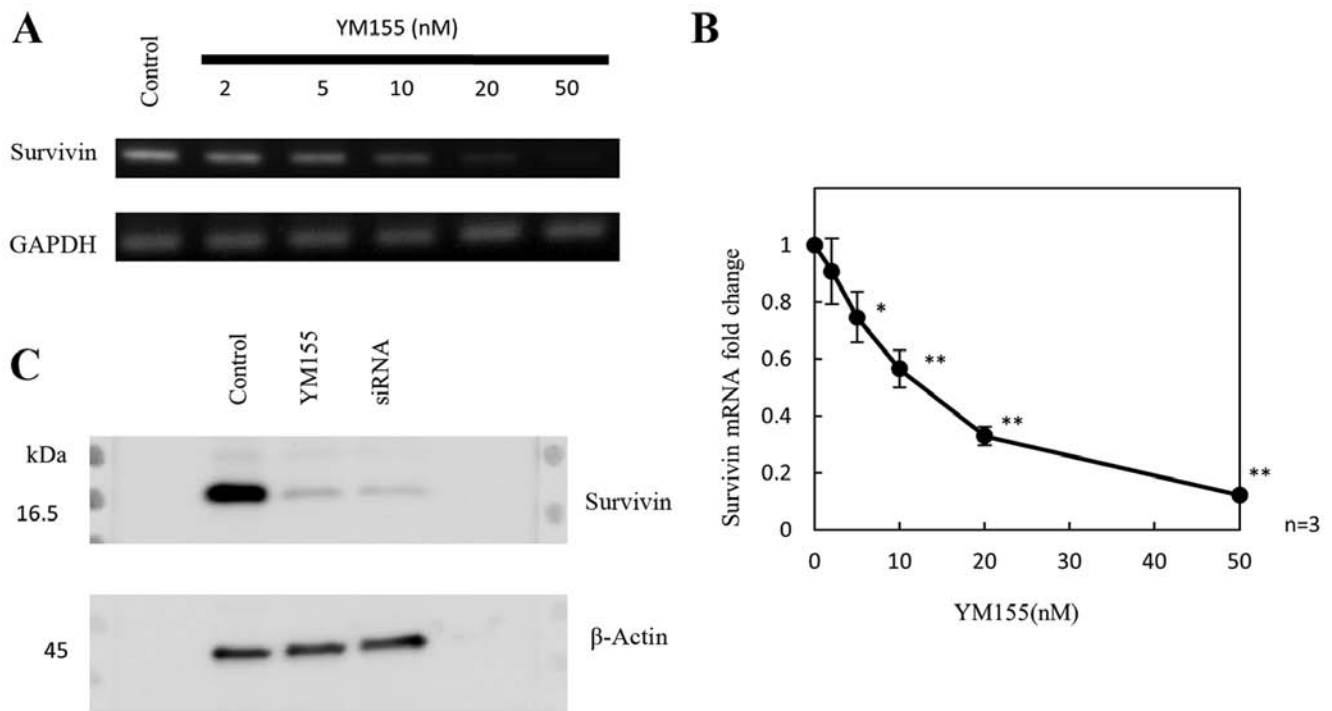
Figure 1. YM155 suppresses survivin expression in human RD cells. Survivin expression decreased after 24 h of treatment with 2-50 nM of YM155 in a dose-dependent manner. (A) Survivin mRNA expression was measured by quantative real-time PCR. YM155 doses of 5 nM and greater suppress survivin mRNA compared to YM155 negative control (*P<0.005, **P<0.001). (B) YM155 suppresses survivin protein in human RD cells as well as siRNA of survivin (C).

purchased from Oriental Yeast. After tumor diameter reached 8 mm, 5 mg/kg of YM155 was administered intraperitoneally (i.p.) once a day for 4 weeks. Cisplatin was administered i.p. at a dose of 2.5 mg/kg twice a week. Tumor diameters were measured once a week, and tumor volumes were determined (length x width^2)/2. Observations continued until day 28 after transplantation. The experiments were reviewed and approved by the Animal Experimentation Committee of Osaka University (permit no. 25-058-000), and conducted in accordance with institutional guidelines. All efforts were made to minimize suffering.

Immunochemistry. Tumors were harvested at the end of the experiments (after 28 days) as fixed, paraffin wax-embedded sections. Sections were subsequently dewaxed and rehydrated, and survivin expression was determined by staining with survivin, desmin, and myoD antibodies with the Real Envision detection system (Dako Denmark A/S, Glostrup, Denmark). After specific staining or hematoxylin and eosin staining, sections were dehydrated in alcohol and xylene and subsequently mounted on a slide.

Apoptosis stain. Tumor sections were subjected to terminal deoxynucleotidyl transferase dUTP-biotin nick end-labeling (TUNEL) staining, performed with an *In Situ* Apoptosis Detection kit (Takara) using anti-FITC horseradish peroxidase conjugate.

Statistical analysis. Results are expressed as relative change compared with DMSO controls and are given as the mean ± SD. The statistical differences between groups were tested using a two-tailed Student's t-test. Statistical significance was assumed for P-values <0.05.

Results

YM155 inhibits endogenous survivin expression in RD cells and induces RMS cell apoptosis in vitro. Endogenous survivin mRNA expression in RD, an RMS cell line, was significantly suppressed in a dose-dependent manner by >10 nM YM155 *in vitro*, measured by quantitative PCR (Fig. 1A). YM155 suppressed survivin expression at concentrations of 5 nM and higher (Fig. 1B). These results suggested that the suppression of survivin by YM155 occurs via the transcriptional inhibition of the survivin gene. We also used western blotting to confirm that YM155 suppressed survivin protein synthesis as well as siRNA did (Fig. 1C).

To evaluate the ability of YM155 to induce cell death in RD cells, we treated the cells with YM155 for 24 and 48 h, after which cell viability was assessed with the WST-8 method. YM155 concentrations from 1 to 50 nM significantly decreased the viability of cells in a dose-dependent manner (Fig. 2).

Suppressing survivin mRNA expression by siRNA or YM155 enhances cisplatin-induced apoptosis in vitro. RD cells were treated with cisplatin or the combination of siRNA and cisplatin, and cell viability was then assessed with the WST-8 method to assess the *in vitro* effects on survivin mRNA suppression. Suppressing survivin mRNA with siRNA significantly decreased the viability of cells, suggesting enhancement of cisplatin-induced apoptosis *in vitro* (Fig. 3).
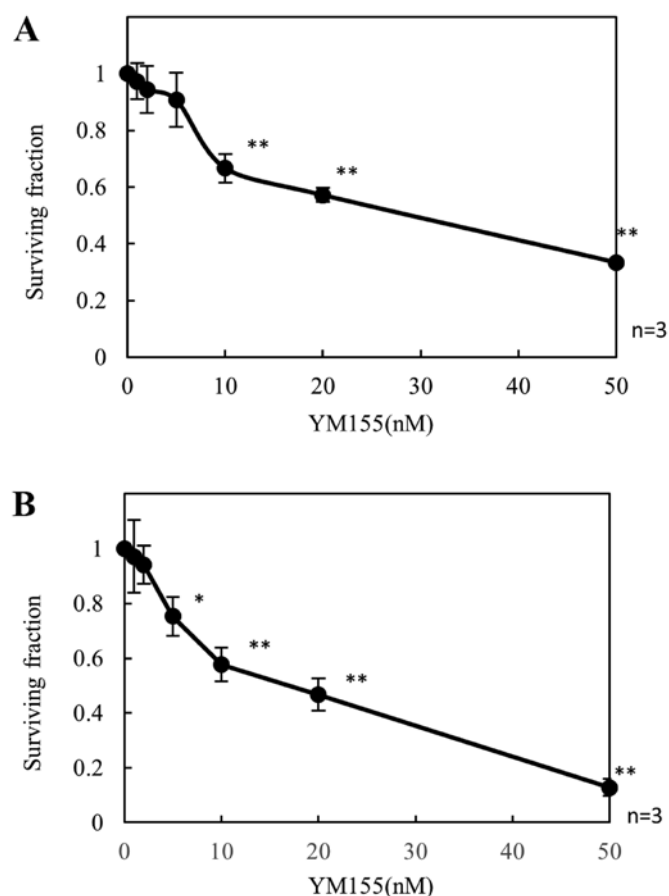
A

B

Figure 2. YM155 inhibits RD cell line growth at 24 h (A) and 48 h (B) incubation in a dose-dependent manner (*P<0.001, **P<0.0001).
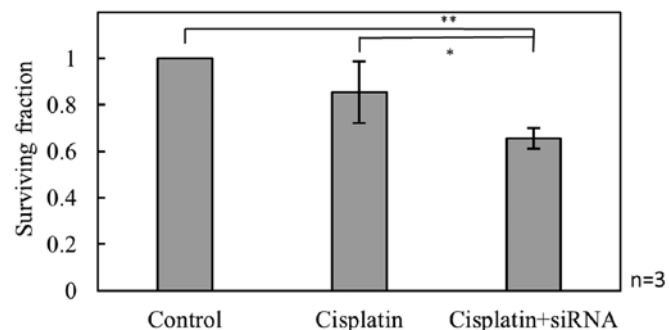

Figure 3. Anti-survivin siRNA inhibits RD cell line growth with 10 µM cisplatin at 24-h incubation (*P=0.02, **P=0.0002).

RD cells were treated with either YM155, cisplatin, or both, after which cell viability was assessed with the WST-8 method to assess the *in vitro* effects on apoptosis induction. At concentrations from 1 to 50 nM, cell viability was significantly decreased in a dose-dependent manner (Fig. 4).

YM155 induces cancer cell apoptosis in vitro. RD cells treated with either YM155, cisplatin, or both were analyzed by Annexin V assay to assess the *in vitro* effects on apoptosis induction (Fig. 5). The number of apoptotic cells was increased by 1.2% by exposure to 10 µM cisplatin (Fig. 5B) and 1.3%
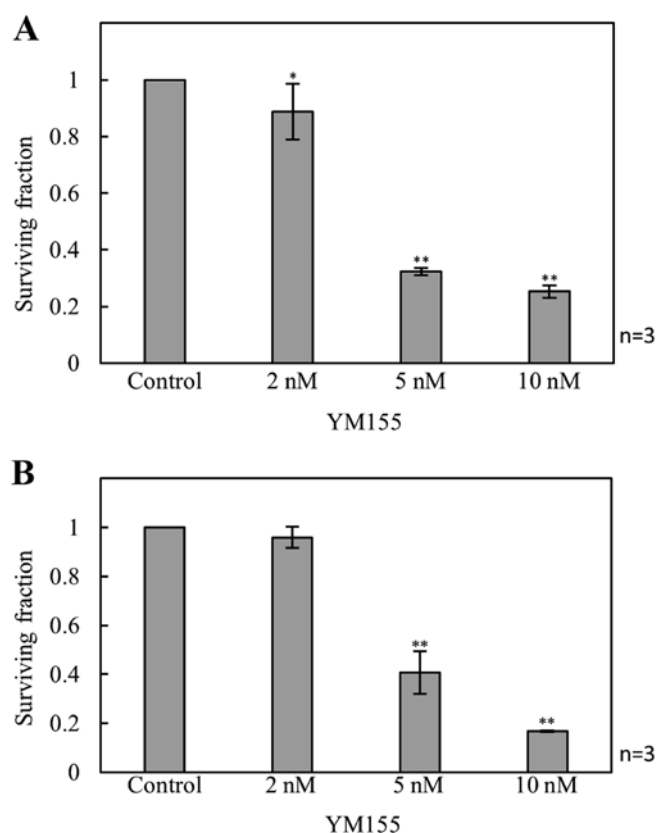
A

B

Figure 4. YM155 inhibits RD cell line growth in combination with 10 µM cisplatin at 24 h (A) and 48 h (B) incubation (*P<0.05, **P<0.0001).

by treatment with 5 nM YM155 (Fig. 5C). In contrast, the combination of cisplatin and YM155 increased the number of apoptotic cells more markedly, by 2% (Fig. 5D). We next examined the effect of YM155 on cisplatin-induced apoptosis in RD cells using the caspase-3 assay. Again, combined treatment of RD cells with YM155 and cisplatin induced a synergistic increase in caspase-3 activity (Fig. 6). These data suggest that YM155 promoted the induction of apoptosis caused by a DNA-damaging agent in the RD cell line.

Antitumor effects of YM155 plus cisplatin in the RD cell xenograft model and immunostaining. We evaluated the *in vivo* antitumor activity of YM155 with i.p. injection into mice xenografted subcutaneously with RD cells as shown in Fig. 7. Mice receiving daily i.p. bolus injections of YM155 at 5.0 mg/kg showed a maximum tumor growth inhibition of 31% compared with control. YM155 showed time-dependent antitumor activity *in vivo*. Mice receiving twice-a-week i.p. injections of cisplatin showed a maximum tumor growth inhibition of 46% compared with control. YM155 in combination with cisplatin significantly inhibited tumor growth by 13% compared with control (P<0.0001). However, each monotherapy suppressed tumor growth, which was followed by an acceleration of growth during the observation period. These results indicate that YM155 in combination with cisplatin was tolerated in mice and enhanced the *in vivo* tumor response to cisplatin (Fig. 7).

Xenograft tumors in all treatment groups were visualized by microscopy *in vivo*, and then resected. Confirming that the
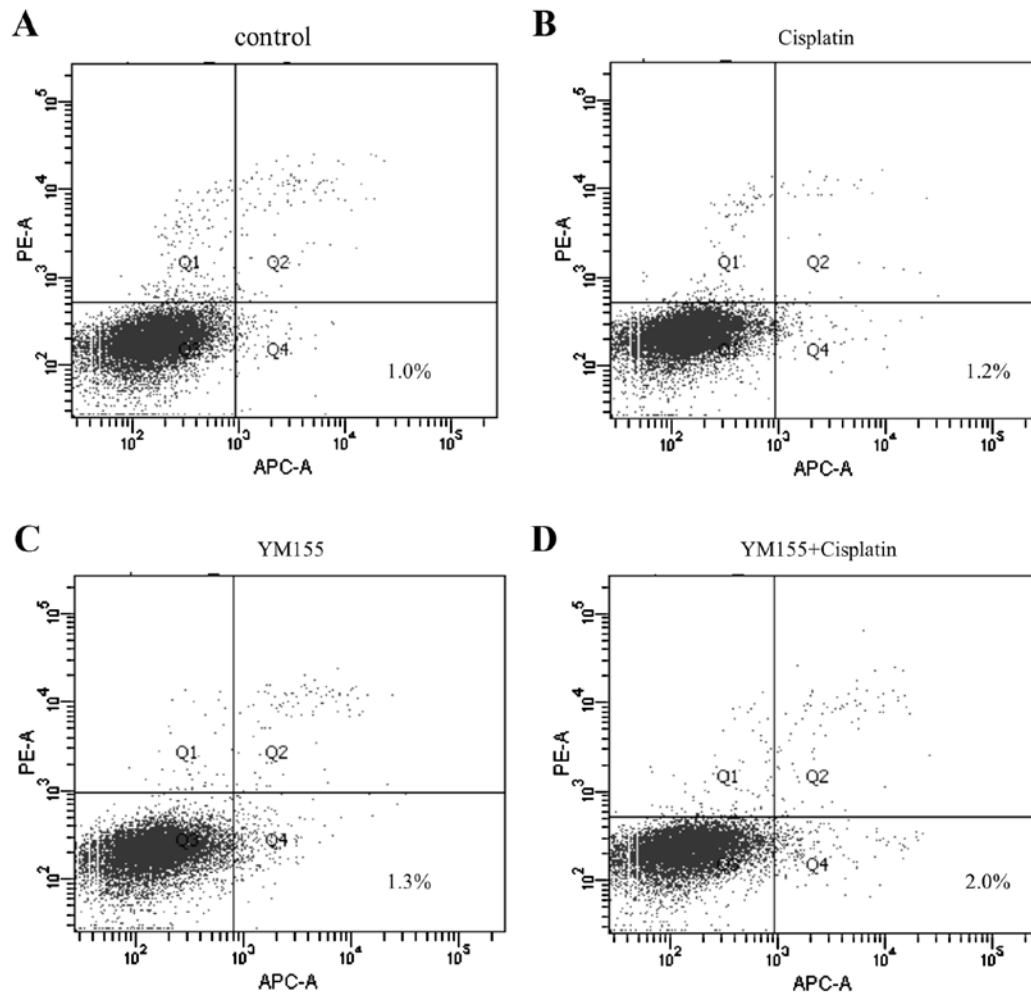
Figure 5. Flow cytometry using Annexin V and propidium iodide (PI). Apoptotic cells were defined as Annexin V-positive and PI-negative (Q4). The combination of YM155 and cisplatin (D) resulted in higher apoptosis than control (A) and either treatment alone (B and C).
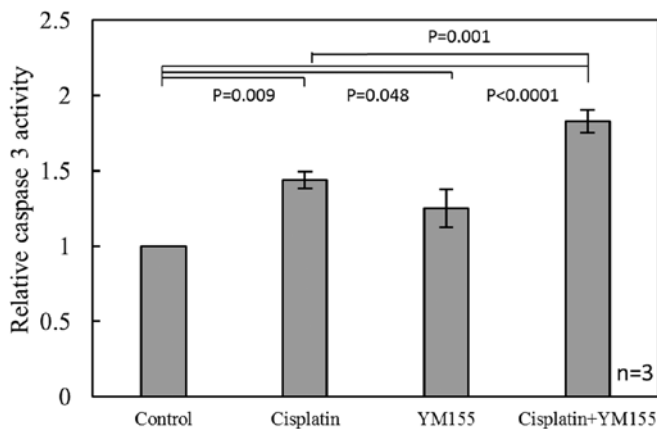

Figure 6. Relative caspase-3 activity following combined treatment with YM155 and cisplatin was greater than with either treatment alone.
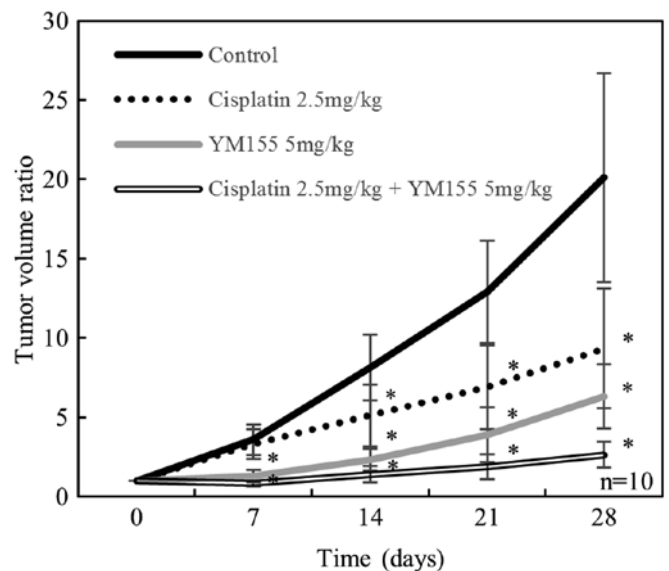

Figure 7. Nude mice (BALB/c nu/nu) subcutaneously injected with RD cells formed palpable tumors. Tumor volume was measured as described in Materials and methods, and the mean tumor volume over the 28-day experimental period was used to generate the graph. The growth rate was calculated during the treatment period. The combination of YM155 and cisplatin resulted in the greatest inhibition of tumor growth (*P<0.0001).

tumors originated from RD cells, immunostaining verified that all specimens expressed the common human myogenic markers desmin and MyoD (Fig. 8A and B). To confirm survivin expression and apoptosis activity, survivin immunostaining and TUNEL staining were performed. Tumors treated with YM155 showed lower levels of survivin than control
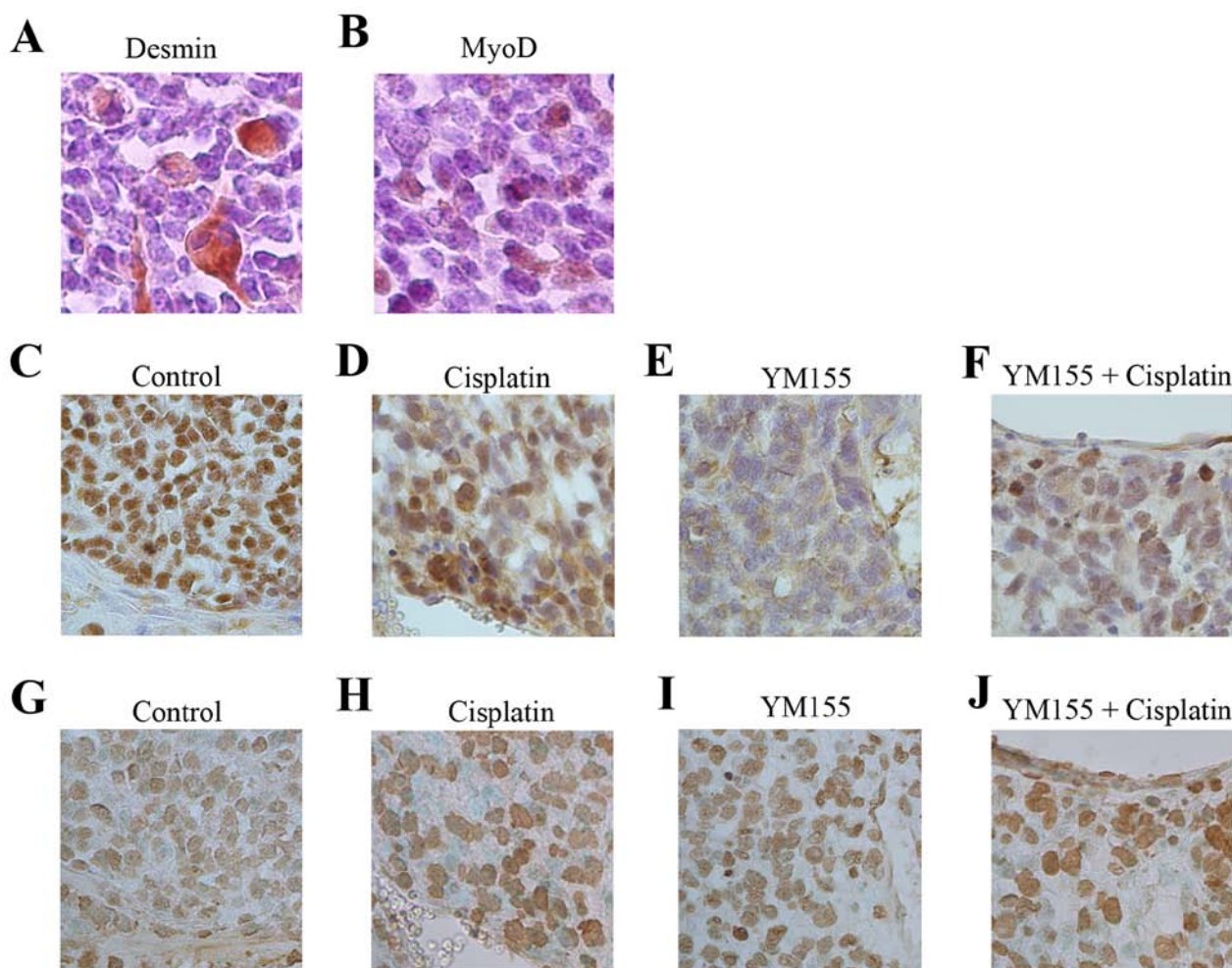
Figure 8. Histology of RMS xenografts. Myogenic markers desmin (A) and myoD (B) expressed in human RMS xenografts. Histology of RMS xenografts. Immunostaining with anti-survivin antibody (E and F). Histology of RMS xenografts. TUNEL staining (G-J).

untreated tumors (Fig. 8C-F). We observed an increase in TUNEL staining in YM155-treated tumors, consistent with the observed growth inhibition of treated tumors (Fig. 8G-J).

Discussion

The mainstay of chemotherapy for RMS is still the VAC regimen. Other drugs are used for second-line treatment and cisplatin has been added in higher risk patients, but to date there is no definitive evidence of improved outcomes with the addition of these drugs, despite evidence of response in the relapse setting (6). The estimated 5-year event-free survival rates in patients with low-, intermediate-, and high-risk disease are 95, 65 and 15%, respectively (32). Therefore, a new therapeutic strategy is critically needed. Here, we examined the therapeutic potential of YM155 alone and in combination with cisplatin in RD cells *in vitro* and in a preclinical RD cell model. We found that YM155 exhibited significant antiproliferative activity and resulted in tumor regression in both RD cells and an established xenograft model.

Survivin is highly expressed in a broad range of solid tumors and hematological malignancies. Increased survivin expression in cancer patients is an unfavorable prognostic marker correlating with decreased overall survival in several malignancies, including lung (33,34), gastric (35), colorectal (18), and breast carcinomas (14), hepatocellular carcinoma (36) as well as in pediatric solid tumors such as neuroblastoma (37), and hepatoblastoma (23). Relationship between survivin expression and RMS are still unknown. However, some anti-apoptotic activities were detected in RMS (38,39). Survivin was strongly overexpressed in both alveolar and embryonal RMS cell lines and in RMS biopsy specimens (40). Blocking survivin expression by RNA interference reduces proliferation and viability of RMS xenotransplants (41). Downregulation of survivin in RMS cells increased their susceptibility toward a T-cell attack (40).

The success of anticancer therapies is often limited by the development of resistance to apoptosis, which may result from defects in common apoptotic pathways (42). In this context, approaches to counteract the action of survivin in tumor cells have been proposed with the dual aims of inhibiting tumor growth through promotion of spontaneous apoptosis and of enhancing the tumor cell response to apoptosis-inducing agents (11). In this study, we found that the combination of YM155 and the platinum compound cisplatin induced RD cell apoptosis as well as the activation of caspase-3 to an extent greater than with either agent alone. Our findings thus suggest that YM155 acts in a synergistic manner to promote the induc-

tion of apoptosis by cisplatin and possibly other platinum compounds.

Survivin was previously shown to enhance tumor cell survival after radiation exposure through regulation of double-strand break repair (43). Iwasa *et al* showed that YM155 inhibited the repair of radiation-induced double-strand breaks in non-small cell lung cancer cells, and that this effect likely accounted for the observed radiosensitizing action of YM155 (44). They suggested that the observed chemosensitisation by YM155 is attributable to inhibition of the DNA damage repair induced by cisplatin (28). Further studies will be required to determine the mechanism underlying the effect of YM155 on DNA repair.

In our *in vitro* study, YM155 synergistically enhanced the antitumor activity of cisplatin in RD cells. In addition, YM155 combined with cisplatin resulted in greater tumor reduction than either treatment alone in an established xenograft model. Taken together, these findings suggest that YM155 may be a promising candidate for RMS therapy as a novel apoptosis inducer with survivin-suppressive activity. Further extensive research on survivin-mediated apoptosis defects may allow for the rational identification of RMS patients who would most likely respond to YM155. Combination chemotherapy is typically employed to achieve a better response rate than monotherapy, and is generally designed empirically using drugs that act through different cytotoxic mechanisms with less overlapping toxicity. Although cisplatin is a second-line drug for RMS, in combination with YM155 it may have a role in the therapy of VAC-resistant patients. Here, we found that YM155 plus cisplatin resulted in more intense apoptosis than either treatment alone. In addition, YM155 potentiated the antitumor activity of cisplatin without exacerbating weight loss in an established xenograft model.

In a toxicological study, short-term exposure to YM155 at high blood concentrations caused cardiotoxicity in the form of atrioventricular block and myocardial degeneration/necrosis, as well as nephrotoxicity, mainly displayed as proximal tubular necrosis and increased serum creatinine. No serious adverse hematological events related to drug treatment were reported in phase I studies of YM155 as single-agent therapy (45). A recent phase II study showed that YM155 monotherapy was safe but only moderately effective in patients with advanced lung cancer (46). Given the limited efficacy of YM155 as a single agent in clinical settings, the combination of YM155 with other agents may be beneficial. Furthermore, YM155 can reduce cisplatin dose in combination chemotherapy. It may enable to decrease serious side effect of cisplatin such as hearing loss, infertility and secondary malignancy. We have shown that the treatment of RD cells with YM155 resulted in a marked increase in the antitumor effects of cisplatin both *in vitro* and *in vivo*, suggesting that the combination of YM155 and platinum compounds may have potential as a novel therapeutic regimen. Clinical studies of YM155 in combination with platinum-based chemotherapies are thus warranted.

In conclusion, long-term chemotherapy is often limited by the development of drug resistance and cumulative side effects. This study showed that YM155 increased the sensitivity of cisplatin by suppressing survivin expression in the embryonal RMS cell line RD. The use of YM155 as an apoptosis inducer, either alone or in combination with cisplatin, may be warranted for the treatment of drug-resistant RMS.

Acknowledgements

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